Rapid Method for the Quantitative Extraction and Simultaneous Class Separation of Milk Lipids

ROBERT J. MAXWELL, DONNA MONDIMORE, and JOSEPH TOBIAS[1]
Eastern Regional Research Center[2]
Philadelphia, PA 19118

ABSTRACT

A method is described for the isolation of lipids from milk, cream, or buttermilk. Lipid isolation is accomplished by solvent elution of a column containing a mixture of the milk product, anhydrous sodium sulfate, and Celite 545. Total lipids are isolated by elution with a 90:10 mixture of dichloromethane:methanol. Alternatively, lipids may be separated into a neutral lipid fraction by sequential elution of the column with dichloromethane and a polar fraction by elution with a 90:10 mixture of dichloromethane:methanol. The proposed method was compared with the traditional Roese-Gottlieb method for a series of milk samples with fat content ranging from <1 to 37%. Fat determinations for a series of five milk types by the dry column and Roese-Gottlieb methods were significantly different for whole raw milk and buttermilk, while means for phospholipids were significantly different for all milk types tested except buttermilk.

INTRODUCTION

Although several methods are now used for isolation of milk lipids (2, 4, 9, 11), the Roese-Gottlieb (Monjonnier) extraction method is used most by dairy scientists to measure the fat content of milk and milk products (8, 11). This method also is employed when lipids are isolated from milk for further chemical analysis. Because certain phospholipids have acidic groups (phosphatidic acid and phosphatidyl serine), recovery of milk phospholipids by extraction from the basic aqueous phase used in the Roese-Gottlieb method would be difficult (2). As alternatives to the Roese-Gottlieb method, modifications of the Folch chloroform:methanol extraction procedure (9) have been proposed. Although lipid recovery is believed to be quantitative in this case, procedural problems are often encountered. Bovine milk in the presence of the chloroform:methanol solvent often forms troublesome emulsions that are very difficult to break. This problem is apparently magnified when human milk is extracted by the Folch technique (4).

To overcome some of the difficulties encountered with traditional milk lipid extraction techniques, we have developed an alternative system for lipid isolation, which obviates some of the problems cited. This method is based on a system originally developed for isolation of lipids from muscle tissue (5, 7) that allows for the quantitative recovery of neutral and polar lipids. Problems such as emulsions are not encountered. Additionally, by a change in the sequence of solvents used to elute the lipids, separate neutral and polar lipid fractions may be isolated in less that 2 h. Because phospholipids are only about 1% of the lipids of whole milk and must ordinarily be recovered by lengthy techniques such as fractionation by silicic acid chromatography, this development represents an important advantage over other methods.

MATERIALS AND METHODS

The various milk products used in each study were prepared from single raw milk samples separated in the Eastern Regional Research Center dairy pilot plant. Separate milk samples used in other studies (Table 1) were purchased at local markets. Prior to analysis, milk samples were heated to 40°C in a water bath to ensure uniform fat distribution. Samples were then weighed into either Mojonnier flasks for the Roese-Gottlieb method (11) or into mortars for the dry column meth-

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[1] Retired, University of Illinois, Urbana 61810. Dairy Research Foundation Fellow at Eastern Regional Research Center.

[2] Agricultural Research Service, US Department of Agriculture.

TABLE 1. Sample: Celite 545: Na_2SO_4 ratios required for milks of varying fat content.

	% Fat	Sample size[1]	Celite 545[2]
		(g)	
Heavy cream	30-40	2	10
Light cream	10-16	2	10
Whole milk	3-4	5	10
Buttermilk	.5-3	5	10
Low fat milk	>1	5	15
Skim milk	<1	5	7.5

[1] For 5-g samples use a 30 cm X 30 mm i.d. glass chromatography column with a 8 cm X 5 mm i.d. drip tip; 2-g samples require 25 mm i.d. columns with similar overall dimensions.

[2] Use 20 g granular anhydrous Na_2SO_4 for all determinations in this table.

od. Sample sizes for the Mojonnier analysis were those specified in the Manual (8). In all comparative tests between the Roese-Gottlieb and dry column methods, quadruplicate analyses were carried out. Phosphorus analysis for each set of Roese-Gottlieb and dry column samples were determined simultaneously using the method of Vaskovsky (10). Some of the same sets were also examined for the presence of free fatty acids by the method of Williams and Macgee (12).

Materials needed for the column analysis are dichloromethane (DCM) and methanol (MeOH) (Burdick and Jackson Laboratories, Muskegon, MI),[3] $CaHPO_4 \cdot 2H_2O$ and Celite 545, not acid washed, Catalog Number C-212 (Fisher Scientific Company, King of Prussia, PA), and granular anhydrous sodium sulfate (Na_2SO_4) (Mallinckrodt, Inc., Paris, KY). A 750-ml mortar, pestle, glass chromatographic columns (see Table 1), and a tamping rod are also needed.

Dry Column Extraction Procedure

A glass wool plug is placed loosely in the drip tip of the chromatography column, and the column is charged with 5 g of a previously prepared mixture of Celite 545 and $CaHPO_4 \cdot 2H_2O$ (9:1), tamped firmly in place. A preweighed container (150-ml tall form beaker) or volumetric flask is used to collect the effluent.

Milk sample either 2 or 5 g (see Table 1) was weighed and transferred to mortar and weight of sample obtained to nearest .1 mg by difference. Twenty grams anhydrous Na_2SO_4 was added and immediately mixed thoroughly with the pestle and a stainless steel spoon to loosen material from mortar walls. Then, Celite 545 (Table 1) was added and quickly ground until a uniform free flowing powder was formed. The resultant mixture was transferred to the column through a powder funnel and firmly tamped in place. Finally, an appropriate flask or beaker was placed under column drip tip and one of the two methods listed next was used for lipid recovery.

Total Lipid Extraction

Spoon and pestle were rinsed over mortar, then the mortar walls, with about 25 ml of a mixture of DCM:MeOH (90:10) by use of a large-volume disposable pipette and was transferred to the inner column wall. Solvent should be allowed to pass through the column bed until eluent appears at drip tip (use more of the 90:10 solvent mixture as needed); then 150 ml of the DCM:MeOH should be added without disturbing column packing, and the column allowed to drip until dry. Collected eluate will contain the total extractable lipids.

Sequential Extraction of Neutral and Polar Lipids

Mortar, pestle, and spoon should be rinsed with about 25 ml of DCM only and added to the column after column preparation. When solvent reaches the column drip tip an additional 100 ml of DCM should be added and let drip until the solvent level is about 1 cm above the column bed. The receiver, which now contains the neutral lipids, should be removed and a second receiver placed under drip tip. The column is then charged with 150 ml of DCM:MeOH (90:10) and allowed to drip until dry. This second receiver now contains the polar lipids. Total elapsed time needed to collect both fractions should range from 90 to 120 min.

RESULTS AND DISCUSSION

Initial experiments with the dry column extraction method were directed toward the isolation of lipids from meat and specific muscle tissues (5, 7). The method also was successfully applied to the lipid analysis of

peanuts (1) and pet foods (6). Because of these encouraging results, we adapted the method to the analysis of lipids in milk. Modifications to the original method were needed to obtain complete recovery of lipids from milk. Whereas extraction of lipids from muscle tissue could be carried out with a fixed ratio of sample: Na_2SO_4:Celite 545 regardless of the fat content. (7), a set ratio was not possible when various milk products were tested by the column method. Instead, the ratio of reagents to sample must be varied according to the fat ranges, as shown in Table 1. Second, it is imperative that the mixing steps be carried out quickly, since the mixture of milk:Na_2SO_4 will tend to harden unless mixing occurs soon after the addition of Na_2SO_4 to the mortar. With these alterations, lipid recoveries improved, and results were reproducible. One problem observed with some column extraction techniques has been that of nonlipid carryover into the lipid extracts; this generally necessitates additional steps in the procedure to eliminate such contaminants (2). In our original beef tissue experiments, this problem was resolved by adding a reagent trap at the base of the column consisting of Celite 545 and $CaHPO_4 \cdot 2H_2O$ (7), which completely retarded the coelution of nonlipid artifacts. This same trap material proved equally effective in tests with milk and thus obviated the need for additional procedures to eliminate nonlipid contaminants from the resultant lipid extracts. Additionally, because of the vapor pressure of DCM, it is advisable to carry out these extractions at temperatures below 26°C.

Milk samples of 2 g and cream samples of 5 g (Table 1) were used in all of the tests described with the dry column method although smaller size samples have been used with other tissues. For instance, in earlier studies with bovine tissue, recoveries of lipid were equivalent from either 1 or 5-g samples (5). Although sample size reduction has not been investigated in the present work for bovine milk, Hundrieser et al. (3) have reported satisfactory results using the dry column method with 1-ml portions of human milk. Reduction in sample size requires a concomitant reduction in the amounts of reagents used and smaller diameter chromatographic columns (6). Additionally, the ratio of sample size to reagents should be adjusted to

TABLE 2. Determination of total fat by the dry column and Roese-Gottlieb methods, and phospholipid content of fat extracts.[1]

Sample	Dry column[2]				Roese-Gottlieb[3]			
	Total fat[4]		Phospholipid[4,5]		Total fat[4]		Phospholipid[4,5]	
	(%)							
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Skim milk	.083	.004[6]	.018	.002	.072	.011[6]	.015	.001
Buttermilk[7]	1.84	.060	.133	.01[6]	1.43	.10	.123	.02[6]
Whole raw milk	4.41	.07	.039	.002	4.07	.11	.030	.002
Light cream	17.03	1.11[6]	.119	.02	16.87	.47[6]	.087	.01
Heavy cream	36.92	1.09[6]	.192	.02	35.82	.32[6]	.165	.01

[1] All milks in table from same whole raw milk source.

[2] Isocratic elution with DCM/MeOH (90/10).

[3] Modification of Walstra and DeGraaf (11).

[4] Mean of four subsamples ± standard deviation.

[5] Calculated as 25 × % phosphorus by the method of Vaskovsky et al. (10).

[6] Within sample type, (for instance dry column total fat vs. Roese-Gottlieb total fat, etc) values are not significantly different by Student's *t* test.

[7] Residue after churning sweet cream.

reflect the fat content of the milk products to be extracted. Regardless of sample size (1 to 5 g), the suggested total height of the packing in the chromatography column should be 7 to 10 cm.

Whole raw milk was separated into component products from heavy cream to skim milk (36.92 to .083% fat), and these products were then extracted by the dry column and Roese-Gottlieb methods (11) for total fat, and for phosphorus content by the method of Vaskovsky et al. (10). Results are shown in Table 2 for lipid recovery by the dry column method compared with Roese-Gottlieb method, where the values shown for total fat were significantly ($P<.05$) greater by dry column method for buttermilk and whole raw milk by Student's t test. Generally, lipid recoveries appear to vary with fat content, a phenomena observed repeatedly with other samples of commercial milks not included in the table. No explanation is apparent for the slightly higher lipid recoveries for the two cited samples by the dry column method. Limited tests also were carried out to determine the presence of free fatty acids in the recovered fat samples (12). In one representative experiment, the free fatty acid contents of the samples from the dry column and Roese-Gottlieb methods were .089 and .072%, respectively, indicating that similar amounts of free fatty acids were recovered by both methods.

Small but significant differences (Student's t test) were observed between the amounts of phospholipid (Table 2) recovered by the two methods for all samples except buttermilk. Absolute differences between the amount of phospholipid extracted by the column and Roese-Gottlieb methods are less for samples with low fat content such as whole milk (.039 vs. .030) or skim milk (.018 vs. . 015) than for the other milks. However, although small, these differences may be important because of the low phospholipid content of whole milk (about 1% or less of the total fat content). Losses of compounds such as phosphatidyl

TABLE 3. Total and sequential extraction of milk lipids by the dry column method.

Sample[1]		Total lipid elution		Sequential elution: Neutral lipid fraction		Sequential elution: Polar lipid fraction		Sum
		(%)						
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	
Milk A	L[2,3]	3.27	.027	3.12	.14	.31	.053	3.43
	PL[3,4]	.028	.002	.00[5]		.037	.011	
Milk B	L	3.81	.027	3.48	.037	.29	.048	3.77
	PL	.027	.003	.00[5]		.025	.003	
Milk C	L	3.63	.110	3.25	.09	.32	.02	3.57
	PL	.015	.001	.00[5]		.013	.001	
Buttermilk A	L	1.90	.050	1.49	.031	.37	.047	1.86
	PL	.122	.001	.00[5]		.127	.003	
Buttermilk B	L	1.21	.030	1.02	.07	.160	.030	1.18
	PL	.038	.001	.00[5]		.034	.003	

[1] All milk samples were whole, raw milk; buttermilk was prepared in the Eastern Regional Research Center dairy pilot plant from raw milk and immediately analyzed.

[2] L = Extractable lipid by either total or sequential methods.

[3] Mean of four subsamples ± standard devaition.

[4] PL = Phospholipid, calculated as 25 X percent phosphorus, determined by the method of Vaskovsky et al. (10).

[5] All neutral lipid fractions were devoid of phospholipids.

serine, for example, may occur when the Roese-Gottlieb method is used for lipid extraction (2) and may partially account for some of the differences observed.

The difficulty of separating the small polar lipid fraction in milk from the total lipid extract by conventional techniques illustrates another aspect of the general utility of the dry column method, for in addition to the extraction of a total lipid extract (Table 2), lipids may be isolated sequentially as separate neutral and polar fractions (Table 3). Additionally, regardless of whether lipids were isolated by the total or sequential methods, recoveries of glycerides and phospholipids were similar (Table 3). Initial extraction of the dry column with DCM alone, for example, yielded a neutral fraction containing the unsaponifiables and glycerides. Upon further sequential elution of the same column with DCM:MeOH, the polar lipid fraction was obtained that contained phospholipids, other polar lipids, and small amounts of residual neutral lipids (5). Phospholipid content of the polar lipid extracts ranged from .013 to .127%. Thus, the dry column method offers a rapid quantitative approach to the difficult problem of milk lipid isolation and provides an extremely simple manner of preparing polar lipid concentrates.

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